ZOOLOGICAL SCIENCE 8: 693–698 (1991)

[COMMUNICATION]

N-Acetyl-D-Galactosamine-Specific Hemagglutinin Purified from Seminal Plasma of the Sea Urchin, *Hemicentrotus pulcherrimus*

Hideki Watanabe, Yuuji Seike, Toshiyuki Miyata[1] and Takashi Suyemitsu

Department of Regulation Biology, Faculty of Science, Saitama University, Urawa 338,
[1]Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, Japan

ABSTRACT—Hemagglutinating activity for human type A erythrocytes was detected in seminal plasma of the sea urchin, *Hemicentrotus pulcherrimus*. Only N-acetyl-D-galactosamine showed any inhibitory effect against the hemagglutinating activity of the seminal plasma. The hemagglutinating activity required calcium ions. This hemagglutinin was purified by affinity chromatography and high-performance liquid chromatography on a TSKgel G3000SW column. A single band of protein was obtained after SDS-polyacrylamide gel electrophoresis of the purified hemagglutinin, corresponding to an apparent molecular weight of 12,000. The amino acid composition of the hemmaglutinin was determined.

INTRODUCTION

Two hemagglutinins have been purified from spermatozoa or seminal plasma of sea urchins. One of these, bindin, was purified from spermatozoa of the sea urchins *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus* [1–3]. The agglutinating activity of bindin for erythrocytes is inhibited by galactose [3], while that for sea urchin eggs is inhibited by sulfated fucans [4]. The second hemagglutinin is HPSPH I, which was purified from seminal plasma of the sea urchin, *Hemicentrotus pulcherrimus*. The agglutinating activity of this material for human type A erythrocytes is inhibited by D-galactose and N-acetyl-D-galactosamine at relatively high concentrations (9.4 mM) [5].

In the present report, we describe the purification of an N-acetyl-D-galactosamine-specific hemagglutinin from seminal plasma of the sea urchin, *Hemicentrotus pulcherrimus*.

Accepted March 15, 1991
Received

MATERIALS AND METHODS

Preparation of seminal plasma

Semen of the sea urchin, *Hemicentrotus pulcherrimus* was obtained by introducing 0.5 M KCl into coelom. Semen was filtered through four layers of gauze and centrifuged at 7,000×g for 10 min. The supernatant was collected as seminal plasma.

Purification of hemagglutinin

Twenty-five ml of seminal plasma were diluted with 9 volumes of 0.055 M NaCl solution and applied to a column of Sepharose-N-caproyl-galactosamine (13 mm i.d.×60 mm), prepared according to the method described by Allen and Neuberger [6] and equilibrated with 0.1 M NaCl, 1 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0 (EB). The column was washed with 100 ml of EB and then

the hemagglutinin was eluted with 30 ml of 0.1 M N-acetyl-D-galactosamine in EB. The eluate was monitored by measurements of the absorbance at 280 nm. The eluate was dialyzed against distilled water, lyophilized, solubilized in 0.2 M phosphate buffer (pH 6.8) and subjected to further purification on a JASCO HPLC system equipped with a JASCO BIP-I pump (Japan Spectroscopic Co., Tokyo). The sample was applied to a TSKgel G3000SW column (7.5 mm i.d. $\times$600 mm) purchased from Tosoh (Tokyo), equilibrated with 0.2 M phosphate buffer (pH 6.8). The sample was eulted at a rate of 0.5 ml/min and the eluate was monitored by measurements of the absorbance at 280 nm. Protein was determined by the method of Lowry *et al.* [7] with bovine serum albumin as a standard.

Assay for hemagglutinating activity

Hemagglutinating activity was determined by serial 2-fold dilutions in microtiter U-plates. The maximum dilution at which hemagglutination occurred was taken as one unit of the hemagglutination titer. For assays of hemagglutinating activity, 25 μl of 0.15 M NaCl and 1 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0 (TBS), and 25 μl of a 2% suspension (v/v) of a trypsin-treated and formalin-fixed human type A erythrocytes in TBS were added to 25 μl of the sample in TBS. The plates were kept at room temperature for 1 hr.

The inhibition assay was carried out in the same microtiter U-plates, as follows. Twenty-five μl of TBS, containing 2 units of the seminal plasma hemagglutinin, were added to 25-μl aliquots of a 2-fold serial dilution of solutions of various sugars in TBS, to which 25 μl of a suspension of human type A erythrocytes in TBS were added. The plates were kept at room temperature for 1 hr and then the hemagglutinating activity was examined. Inhibitory activity was expressed as the minimum concentration of each sugar that caused complete inhibition of hemagglutination.

SDS-polyacrylamide gel electrophoresis

Electrophoresis was carried out on 15% polyacrylamide gels in the presence of SDS, by the method of Laemmli [8]. The gels were stained with silver [9]. A molecular marker kit containing fragments of myoglobin (BDH Chemicals Ltd., Poole, U.K.) was used to allow estimation of the molecular weight of the hemagglutinin.

Amino acid analysis

Amino acid analysis of the hemagglutinin was carried out with a reaction liquid chromatography system, model 655 from Hitachi, Ltd. (Tokyo), after hydrolysis of the sample for 24 hr in 6 N HCl at 110°C [10]. Cysteine was determined as cysteic acid after oxidation of the hemagglutinin by performic acid [11].

Chemicals

D-Glucose, D-galactose, D-mannose, L-fucose, L-arabinose, D-xylose and lactose were purchased from Wako Pure Chemicals (Osaka). Methyl-α-D-mannoside was purchased from Fluka Chemie AG (Buchs, Switzerland). D-Glucosamine, D-galactosamine, D-mannosamine, N-acetyl-D-glucosamine and N-acetyl-D-mannosamine were purchased from Nakarai Chemicals (Kyoto). N-Acetyl-D-galactosamine (GalNAc) and N-acetylneuramic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All the other reagents were of either HPLC or analytical grade.

RESULTS

Seminal plasma of the sea urchin, *Hemicentrotus pulcherrimus,* exhibited hemagglutinating activity for trypsin-treated and formalin-fixed human type A erythrocytes, as shown in Table 1. This hemagglutinating activity was inhibited by the addition of 8 μM N-acetyl-D-galactosamine. The other sugars tested in this system, namely, D-glucose, D-galactose, D-mannose, methyl-α-D-mannoside, L-fucose, D-glucosamine, D-galactosamine, D-mannosamine, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, N-acetylneuramic acid and lactose, did not inhibit the hemagglutinating activity when present at concentrations of 3.3 mM. When Ca^{2+} was absent from the reaction mixture, no hemagglutinating activity was detected. The activity appeared when the concentration of Ca^{2+} was increased to 50 μM. Mg^{2+} had no effect on the hemagglutinating activity, even at concentrations as high as 3.3 mM. When the seminal plasma was

Table 1. Purification of hemagglutinins from seminal plasma

Fraction	Total amount of protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield of activity (%)
Seminal plasma	24	128,000	5,300	100
Eluted from affinity column	0.5	50,000	100,000	39
Separated by HPLC				
Fr. A	0.19	11,800	61,000	9
Fr. B	0.21	26,000	120,000	20

boiled for 30 sec, 75% of the total hemagglutinating activity was lost.

The diluted seminal plasma was applied to a column of Sepharose-N-caproylgalactosamine. Figure 1 shows the elution profile. All fractions were collected and concentrated, and their activities were assayed. Most of hemagglutinating activity was concentrated in the fraction under the bar in Figure 1. Affinity chromatography allowed recovery of 39% of the activity of the starting material with a 19-fold purification of the activity, as shown in Table 1.

The active fraction was applied to a TSKgel G3000SW column and separated by HPLC into four fractions, as shown in Figure 2. The hemagglutinating activities of the four fractions were assayed. Only fractions A and B exhibited any hemagglutinating activity, as shown in Table 1. The amount of activity in fraction A was less than that in fraction B and the specific activity of fraction A was lower than that of fraction B. Fraction B contained 20% of the starting activity

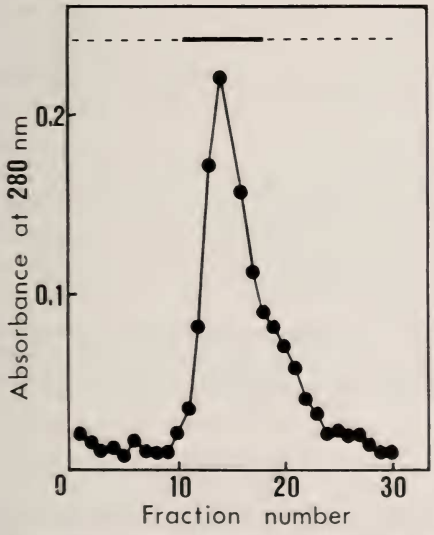

Fig. 1. Elution profile of seminal plasma of the sea urchin, *H. pulcherrimus*, from a column of Sepharose-N-caproylgalactosamine. The diluted seminal plasma was applied to the column, which had been equilibrated with EB. The column was first washed with 100 ml of EB. Then the sample was eluted with 30 ml of 0.1 M GalNAc in EB. One ml of effluent was collected in each tube after the start of the elution with the sugar solution and the absorbance of each fraction at 280 nm was measured. The broken line and the bar represent the fractions whose hemagglutinating activities were assayed.

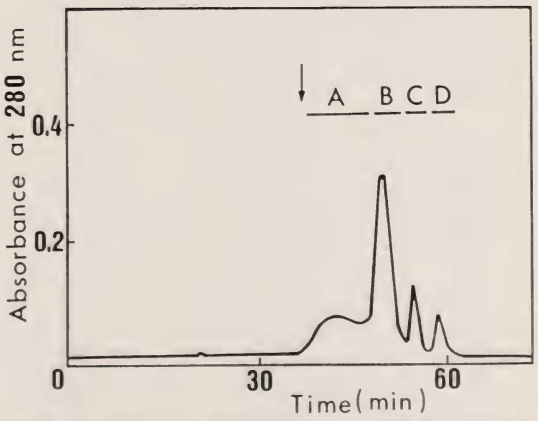

Fig. 2. Purification of the seminal-plasma hemagglutinin by HPLC. The active sample obtained by affinity chromatography was purified by HPLC on a TSKgel G3000SW column which was equilibrated with 0.2 M phosphate buffer (pH 6.8). It was eluted at a rate of 0.5 ml/min and the eluate was monitored by measurements of abosrbance at 280 nm. Arrow indicates the elution position of HPSPH I purified according to Method I described by Yamada and Aketa [5].

with a 23-fold purification, as shown in Table 1.

Single bands of protein were obtained after SDS-PAGE of fractions A and B, respectively, with the same mobility, as shown in Figure 3.

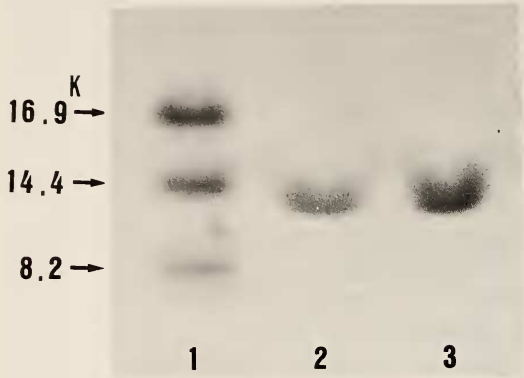

Fig. 3. SDS-PAGE of seminal-plasma hemagglutinin purified by HPLC. Lane 1, standard proteins: myoglobin (Mr 16,949), myoglobin I and II (Mr 14,404), myoglobin I (Mr 8,159). Lane 2, fraction A from HPLC. Lane 3, fraction B from HPLC.

Table 2. Amino acid compositions of hemagglutinins from seminal plasma

Amino acid	Hemagglutinins eluted by HPLC	
	Fraction A	Fraction B
	(mol %)	
Asp	11.1	12.9
Thr	2.6	2.2
Ser	10.4	8.5
Glu	19.2	18.1
Pro	4.1	4.6
Gly	13.3	13.1
Ala	6.7	7.0
Cys[a]	6.6	6.4
Val	3.0	3.6
Met	0.7	0.6
Ile	2.8	3.3
Leu	3.6	3.9
Tyr	2.2	1.7
Phe	3.4	4.2
Lys	4.9	4.8
His	2.7	2.5
Arg	2.7	2.6
Total	100	100

[a] Estimated as cysteic acid after oxidation of the hemagglutinin by performic acid.

They are corresponding to an apparent molecular weight of 12,000.

The amino acid compositions of fractins A and B are given in Table 2. The amino acid composition of fraction A was very similar to that of fraction B. The similarity between fractions A and B in the amino acid compositions and the molecular weights determined by SDS-PAGE suggests that fraction A may represent the aggregated form of fraction B. Sugar specificity was assayed by inhibition of hemagglutination caused by 2 units of fraction B. The results are given in Table 3.

Table 3. Inhibition characteristic of the GalNAc-specific hemagglutinin

Hapten	I (mM)
D-Glucose	>50
D-Galactose	10
D-Mannose	>50
Methyl-α-D-mannoside	>50
L-Arabinose	20
L-Fucose	>50
D-Xylose	>50
D-Glucosamine	>50
D-Galactosamine	>50
D-Mannosamine	>50
N-Acetyl-D-glucosamine	>50
N-Acetyl-D-galactosamine	0.004
N-Acetyl-D-mannosamine	>50
N-Acetylneuramic acid	>50
Lactose	15

I is the concentration of hapten required to inhibit the hemagglutination effected by 2 units of fraction B.

DISCUSSION

The amino acid compositions of the material in fractions A and B are different from that of bindin [2], whose molecular weight was reported to be 30,500. The GalNAc-specific hemagglutinin detected in the seminal plasma can be concluded to be different from bindin because of the differences between the GalNAc-specific hemagglutinin and bindin in their molecular weights, amino acid compositions and sugar specificities [1–3].

The molecular wight of HPSPH I was reported to be about 140,000 and its agglutinating activity is

inhibited by D-galactose and GalNAc [5]. When ammonium sulfate precipitate of seminal plasma, fractionated according to the method of Yamada and Aketa [5], was applied to the column of Sepharose-N-caproyl-galactosamine, HPSPH I did not bind to the column under the condition described in Materials and Methods (data not shown). Moreover, when HPSPH I, purified according to Method I described by Yamada and Aketa [5], was applied to the TSKgel G3000SW column, it eluted at the different position from those of the GalNAc-specific hemagglutinins, as shown in Figure 2. All these results, as well as the differences between the GalNAC-specific hemagglutinin and HPSPH I in their molecular weights and sugar specificities, suggest that the GalNAc-specific hemagglutinin reported in the present study is different from HPSPH I.

The coelomic-fluid hemagglutinin has been purified from the sea urchin, *Anthocidaris crassispina* [12, 13]. However, the coelomic fluid of *H. pulcherrimus* exhibited no hemagglutinating activity for human hype A erythrocytes. Therefore, the hemagglutinating activity in the seminal plasma was not due to contamination by the coelomic fluid.

Many animal lectins have been documented; they play important roles in recognition processes, including those involved in adhesion between cells [14]. They can be roughly separated into two groups: the C-type (Ca^{2+}-dependent) animal lectins and the S-type (thiol-dependent) animal lectins. The C-type animal lectins require Ca^{2+} for activity and their usual location is extracellular [14]. The GalNAc-specific hemagglutinin in the seminal plasma of *H. pulcherrimus* requires Ca^{2+} and its location is extracellular. Therefore, the GalNAc-specific hemagglutinin can be assigned to the group of the C-type animal lectins.

It is not likely that the hemagglutinin in the seminal plasma plays a significant role in the process of fertilization of sea urchin eggs, because the semen is rapidly diluted after it is spawned into sea water. Therefore, a biological role for the hemagglutinin remains to be established.

ACKNOWLEDGMENTS

The authors wish to express their thanks to Dr. K. Ishihara for reading through the manuscript.

REFERENCES

1 Vacquier, V. D. and Moy, G. W. (1977) Isolation of bindin: The protein responsible for adhesion of sperm to sea urchin eggs. Proc. Natl. Acad. Sci. U.S.A., **74**: 2456–3450.

2 Bellet, N. F., Vacquier, J. P. and Vecquier, V. D. (1977) Characterization and comparison of "bindin" isolated from sperm of two species of sea urchins. Biochim. Biophys. Res. Comm., **79**: 159–165.

3 Vacquier, V. D. and Moy, G. W. (1978) Macromolecules mediating sperm-egg recognition and adhesion during sea urchin fertilization. In "ICN-UCLA Symposia on Molecular and Cellular Biology". Ed. by E. R. Dirksen, D. M. Prescott and C. F. Fox, Academic Press, New York, pp. 379–389.

4 DeAngelis, P. L. and Glabe, C. G. (1987) Polysaccharide structural features that are critical for the binding of sulfated fucans to bindin, the adhesive protein from sea urchin sperm. J. Biol. Chem., **262**: 13946–13952.

5 Yamada, Y. and Aketa, K. (1982) Purification and partial characterization of hemagglutinins in seminal plasma of the sea urchin, *Hemicentrotus pulcherrimus*. Biochim. Biophys. Acta, **709**: 220–226.

6 Allen, A. K. and Neuberger, A. (1975) A simple method for the preparation of an affinity absorbent for soybean agglutinin using galactosamine and CH-Sepharose. FEBS Lett., **50**: 362–364.

7 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem., **193**: 265–175.

8 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**: 680–685.

9 Oakley, B. R., Kirsh, D. R. and Morris, N. R. (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem., **105**: 361–363.

10 Benson, J. R. and Hare, P. E. (1975) *o*-Phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. Proc. Natl. Acad. Sci. U.S.A., **72**: 619–622.

11 Moore, S. (1963) On the determination of cysteine as cysteic acid. J. Biol. Chem., **238**: 235–237.

12 Giga, Y. and Ikai, A. (1985) A new multimeric hemagglutinin from the coelomic fluid of the sea urchin *Anthocidaris crassispina*. Biochemistry, **24**: 4461–4467.

13 Giga, Y., Ikai, A. and Takahashi, K. (1987) The complete amino acid sequence of echinoidin, a lectin from the coelomic fluid of the sea urchin *Anthocidaris crassispina*. J. Biol. Chem., **262**: 6197–6203.

14 Drickamer, K. (1988) Two distinct classes of carbohydrate-recognition domains in animal lectins. J. Biol. Chem., **263**: 9557–9560.